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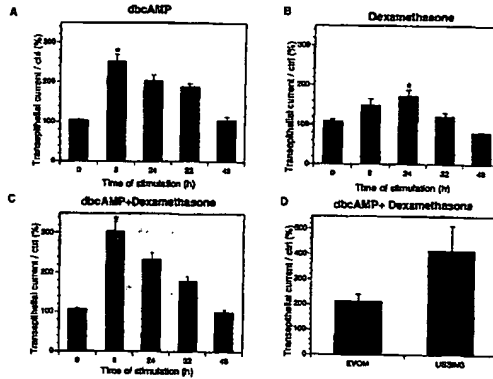
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(54) CONSTRUCTIONS COMPRENANT LE PROMOTEUR DU CANAL SODIQUE EPITHELIAL ET UN GENE  
RAPPORTEUR POUR LE CRIBLAGE DE PRODUITS PHARMACEUTIQUES

(54) CONSTRUCTS COMPRISING THE ENAC PROMOTER SEQUENCE AND A REPORTER GENE FOR  
SCREENING PHARMACEUTICALS

(57)

This invention relates to an epithelial sodium channel gene promoter. This promoter is operably linked to a reporter gene, and used in the screening of compounds for treating diseases or disorders involving epithelial sodium abnormal activity and consequent liquid retention.



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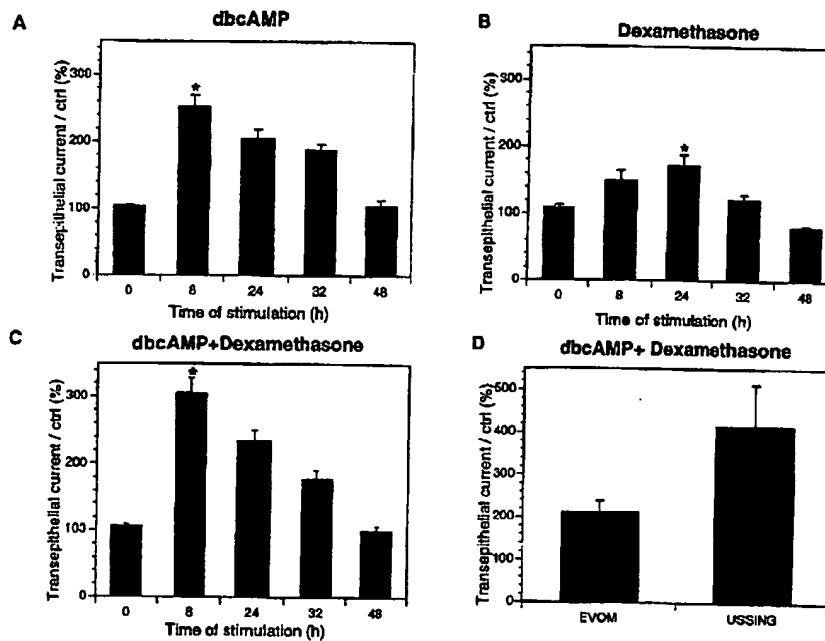
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(57) Abrégé/Abstract:

This invention relates to an epithelial sodium channel gene promoter. This promoter is operably linked to a reporter gene, and used in the screening of compounds for treating diseases or disorders involving epithelial sodium abnormal activity and consequent liquid retention.

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**ABSTRACT OF THE DISCLOSURE**

This invention relates to an epithelial sodium channel gene promoter. This promoter is operably linked to a reporter gene, and used in the  
5. screening of compounds for treating diseases or disorders involving epithelial sodium abnormal activity and consequent liquid retention.

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**TITLE OF THE INVENTION**

Constructs comprising the ENaC promoter sequence and a reporter gene for screening pharmaceuticals.

**FIELD OF THE INVENTION**

This invention relates to constructs for screening valuable active compounds, using a reporter gene operably linked to a gene promoter, namely a sodium channel gene promoter.

**BACKGROUND OF THE INVENTION**

Vectorial sodium transport from alveoli to the interstitium is the main mechanism by which alveolar epithelial cells regulate the amount of fluid lining the alveoli. Sodium entering by the amiloride-sensitive Na<sup>+</sup> channel (ENaC) located at the apical surface of these cells is extruded by the sodium pump (Na<sup>+</sup>-K<sup>+</sup>-ATPase) located at the basolateral surface (39). The osmotic gradient created by this transport is the main driving force involved in the clearance of liquid in adult lungs (4;39) and plays an important role at birth in the removal of fetal lung liquid (48). Recent experimental data have allowed us to better define the structure and function of the major system involved in this transepithelial transport.

ENaC is an important constituent of the Na<sup>+</sup> transport system. The channel has been cloned recently (8;9;21;41;75), and is constituted of 3 subunits,  $\alpha$ ,  $\beta$  and  $\gamma$ ENaC (9). In situ hybridization and immunohistochemical staining have shown that ENaC subunits are expressed along the epithelium of the respiratory system (7;24;38;56) and are detected in alveolar type II cells (24;38). The physiological role of  $\alpha$ ENaC in the lung has been demonstrated in a mouse model where the  $\alpha$ ENaC gene was deleted by targeting a transgene by homologous recombination (33). Unable

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to clear liquid from their lungs, these mice die shortly after birth (33).

The other major component of the transepithelial  $\text{Na}^+$  transport system is  $\text{Na}^+\text{-K}^+\text{-ATPase}$  which is constituted of 2 subunits. The  $\alpha$  subunit, the catalytic component of the complex, is involved in  $\text{Na}^+$  extrusion,  $\text{K}^+$  intrusion and ATPase activity (22). The  $\beta$  subunit is a highly glycosylated protein whose role is not well understood but it seems to be an important regulatory component of the sodium pump (22). The  $\alpha_1$  and  $\beta_1$  subunits are isoforms that have been detected in the lungs (50;52). Inhibition of  $\text{Na}^+\text{-K}^+\text{-ATPase}$  with ouabain has been shown to greatly reduce solute transport in alveoli (3) and the short circuit current  $I_{\text{SC}}$  of alveolar type II cells (15).

Several studies have revealed that pharmacological or physiological rises in the sodium transport ability of the distal lung are associated with the heightened expression of various components of the sodium transport system. Modulation of  $\alpha\text{ENaC}$  and  $\alpha\text{Na}^+\text{-K}^+\text{-ATPase}$  mRNA has been reported at birth and during lung development (20;48;74) when sodium transport allows clearance of the alveolar spaces (48). There is also an increase of  $\alpha_1\text{Na}^+\text{-K}^+\text{-ATPase}$  protein expression during the resolution of pulmonary edema (79). Hyperoxia and hypoxia modulate the expression of  $\alpha\text{ENaC}$  and  $\text{Na}^+\text{-K}^+\text{-ATPase}$  mRNA in lungs (30;45;77) and alveolar epithelial cells (10;54;55;77). Collectively, all these data suggest that changes in  $\alpha\text{ENaC}$  and  $\alpha_1\text{Na}^+\text{-K}^+\text{-ATPase}$  expression could play a role in the altered sodium transport and lung liquid clearance.

We observed recently that sustained treatment of alveolar type II cells with the  $\beta$ -agonist terbutaline enhances  $\alpha\text{ENaC}$  and  $\text{Na}^+\text{-K}^+\text{-ATPase}$  expression (43), suggesting that cAMP could be involved in their regulation. Dexamethasone, a synthetic steroid, is known to increase  $\alpha\text{ENaC}$  mRNA expression in the fetal lung (49;69) and in cultured fetal epithelial cells

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(12) where it raises amiloride-sensitive current (12). Recent findings have shown that functional glucocorticoid regulatory elements (GRE) are present in the promoter of  $\alpha$ ENaC (53),(63) indicating that dexamethasone could act on ENaC-mediated sodium transport by affecting the expression of the channel at the gene level.

Although modification of ENaC and  $\text{Na}^+$ - $\text{K}^+$ -ATPase gene expression could be important for lung liquid clearance, the exact regulatory mechanisms involved are still unknown, particularly in adult lung epithelial cells. In the present study, we evaluated the regulation of  $\alpha$ ENaC and  $\alpha_1\text{Na}^+$ - $\text{K}^+$ -ATPase gene expression by cAMP and dexamethasone in alveolar type II cells purified from the adult rat lung. The  $\alpha$ ENaC gene was chosen because it is the only ENaC subunit able to drive sodium transport when expressed alone in *Xenopus laevis* oocytes (8;9). Conversely, we studied  $\alpha_1\text{Na}^+$ - $\text{K}^+$ -ATPase mRNA expression because it codes for the catalytic domain of  $\text{Na}^+$ - $\text{K}^+$ -ATPase (22). After evaluating the time course of  $\alpha$ ENaC and  $\alpha_1\text{Na}^+$ - $\text{K}^+$ -ATPase mRNA following treatment with dibutyryl cAMP (dbcAMP) or dexamethasone, we investigated the role of transcription and translation in the modulation of these genes by exposing the cells to actinomycin D and cycloheximide. Because we found that  $\alpha$ ENaC gene transcription was modulated directly by dbcAMP and dexamethasone, we cloned and sequenced 2.7 kb of mouse  $\alpha$ ENaC 5'-flanking DNA and tested the activity of the promoter in A549 lung epithelial cells. Finally, we assessed the physiological impact of dbcAMP and dexamethasone on transepithelial current generated at times when  $\alpha$ ENaC mRNAs are upregulated.

Because of the important role of sodium transport, there is a need for developing drugs to modulate this transport, and consequently, there is a need for screening drugs for this purpose.

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**SUMMARY OF THE INVENTION**

Other objects, advantages and features of the present invention will become more apparent upon reading of the following non restrictive description of preferred embodiments thereof, given by way of example only with reference to the accompanying drawings.

An object of the present invention is to provide an epithelial sodium channel gene promoter having the nucleic acid sequence shown in Figure 5, a variant thereof or a fragment thereof comprising at least one of regulatory elements responding to at least one DNA-binding proteins shown in Figure 6.

Other objects relate to a DNA construct comprising the promoter and a heterologous gene operably linked thereto, namely a reporter gene, an expression vector comprising that construct, and a recombinant host cell comprising the construct. The recombinant host cell may be an epithelial cell, preferably originating from a tissue involved in sodium reabsorption. Most preferably, the host cell endogenously expresses said sodium channel.

Another object of this invention is to provide a method for screening compounds to select a potentially active pharmaceutical capable of modulating the expression of an epithelial sodium channel gene, which comprises:

- testing said compounds with a recombinant host cell as defined hereinabove;
- detecting a difference between the levels of expression of said reporter gene in the presence and in the absence of said compounds; and
- selecting a compound which shows such a difference as said potential pharmaceutical of interest.

The pharmaceutical that is sought is for treating sodium

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and water retention.

When the host cell is a alveolar lung cell, the pharmaceutical is for treating lung edema, and when the cell host cell is a tubular kidney cell, the pharmaceutical is for treating hypertension, for example.

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1. Modulation of  $\alpha$ ENaC (A,C) and  $\alpha_1$ Na<sup>+</sup>-K<sup>+</sup>-ATPase mRNA (B,D) expression by dbcAMP (A,B) and dexamethasone (C,D) detected by Northern blot quantitation. Alveolar type II cells were cultured for 3 days and then treated for 1 h, 4 h, 8 h, 24 h or 48 h with 1 mM dbcAMP or 100 nM dexamethasone. There was a 2-fold increment in the level of  $\alpha$ ENaC and  $\alpha_1$ Na<sup>+</sup>-K<sup>+</sup>-ATPase mRNA after 8 h of dbcAMP treatment. In dexamethasone-treated cells,  $\alpha$ ENaC mRNA increased gradually to maximum 4.5-fold expression after 24 h (Fig. 1C). The data are presented as percentages of expression  $\pm$  SE relative to time-matched untreated controls. mRNA expression was corrected to  $\beta$ -actin expression. n=number of animals from different experiments. For dbcAMP treatment: 1 h, n=4; 4 h, n=7; 8 h, n=18; 24 h, n=8; 48 h, n=8; for dexamethasone treatment: 1 h, n=10; 4 h, n=13; 8 h, n=14; 24 h, n=10; 48 h, n=10.  $\star p < 0.05$  4 h and 8 h; 8 h and 24 h (A and B); 8 h and 24 h (C) by post hoc comparison (Fisher PLSD).

Figure 2. Representative Northern blot and corresponding densitometric quantitation showing the effect of combined treatment with dbcAMP and Dex on the expression of  $\alpha$ ENaC and  $\alpha_1$ Na<sup>+</sup>-K<sup>+</sup>-ATPase mRNA. Alveolar type II cells were cultured for 3 days and treated for 8 h with 1 mM dbcAMP, 100 nM Dex or a combination of both agents. Combined treatment with dbcAMP and Dex had an additive effect on  $\alpha$ ENaC and  $\alpha_1$ Na<sup>+</sup>-K<sup>+</sup>-ATPase mRNA. The data are presented as percentages of

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expression compared to time-matched controls. The amount of mRNA was corrected with the level of  $\beta$ -actin mRNA. (dbcAMP, n=18; Dex, n=12; dbcAMP + Dex, n=3). dbcAMP: dibutyryl cAMP; Dex: dexamethasone.

Figure 3. Representative Northern blots showing the role of transcription and translation in the modulation of  $\alpha$ ENaC and  $\alpha_1$ Na<sup>+</sup>-K<sup>+</sup>-ATPase mRNA expression following treatment with dbcAMP or dexamethasone. Alveolar cells were treated for 8 h with 1 mM dbcAMP or 100 nM dexamethasone in the presence or absence of the transcription inhibitor actinomycin D (5  $\mu$ g/ml)(A) or the translation inhibitor cycloheximide (2.5  $\mu$ g/ml)(B). Actinomycin D depressed the increase of  $\alpha$ ENaC mRNA detected after treatment with dbcAMP or dexamethasone and inhibited the elevation of  $\alpha_1$ Na<sup>+</sup>-K<sup>+</sup>-ATPase mRNA in dbcAMP-treated cells. Cycloheximide had an impact on  $\alpha$ ENaC mRNA expression, decreasing the basal expression level of the transcripts, but did not inhibit the increase of  $\alpha$ ENaC mRNA evoked by dbcAMP and dexamethasone. Cycloheximide suppressed the elevation of  $\alpha_1$ Na<sup>+</sup>-K<sup>+</sup>-ATPase mRNA elicited by dbcAMP. Ctrl: control; db: dibutyryl cAMP; Act D: actinomycin D; dex: dexamethasone; Cyclo: cycloheximide.

Figure 4.  $\alpha$ ENaC mRNA stability. The half-life of  $\alpha$ ENaC mRNA was determined in control cells (A) and cells treated with 1 mM dibutyryl cAMP (B) or 100 nM dexamethasone (C). The cells were treated with 5  $\mu$ g/ml actinomycin D for 0, 2, 4, 6, 8 or 10 h in the presence or absence of 1 mM dibutyryl cAMP or 100 nM dexamethasone. 18S rRNA was used to normalize the amount of RNA loaded on each well. Four different experiments were combined to generate the mRNA decay slope.

Figure 5. Sequence of 5'-flanking DNA of the mouse  $\alpha$ ENaC gene showing different 5'UTR resulting from different transcription initiation sites and putative transcription factor binding sites defined with the TESS program. Several potential transcription initiation sites were detected

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by 5'-RACE. The sequences are numbered to the more distal transcription initiation site. Arrows show the 2 major transcription initiation sites found (black arrow: lung; open arrow: kidney), and the arrowheads, the minor start site cloned (black arrowhead: lung; open arrowhead: kidney). The first codon is labelled in bold letters (ATG). Putative transcription factor binding sequences are shown in boxes. The Glucocorticoid receptor binding sequences (GR) are underlined. GenBank accession number: AF228802.

Figure 6. Activity of the  $\alpha$ ENaC promoter in A549 cells. (A) Map of the 3-kb  $\alpha$ ENaC mouse genomic clone showing the position of the putative CRE and GRE transcription factor binding sequences. Arrows indicate the 2 major transcription initiation sites detected by 5'-RACE in the lung. The rectangle depicts the part of exon I encompassed in the clone with the 5'UTR (open box) and coding sequence (ORF) (black box). For the reporter gene assay, a BamHI-MscI 2.9-kb fragment was linked upstream of a CAT gene in the p $\beta$ gCAT vector. The genomic fragment ends at the 5'UTR downstream of the first transcription initiation site. (B) Activity of the CAT reporter gene transfected in A549 cells expressed as arbitrary units (cpm of sample -cpm of untransfected cells). The p $\beta$ gCAT vector alone (n=10) has very low background expression. The BamHI-MscI  $\alpha$ ENaC genomic clone is able to drive basal expression of the gene in control untreated cells (CTRL; n=9) and 8 h dbcAMP-treated cells (dbcAMP 8h; n=8). Treatment with dexamethasone for 24 h (Dex 24h; n=10) increases the basal activity of the promoter by a factor of 5.9. P<0.05 ( $\star$  Dex 24h:CTRL) ( $\ast$  CTRL: p $\beta$ gCAT) by unpaired t-test.

Figure 7. Impact of dbcAMP and dexamethasone treatment on transepithelial current. Alveolar type II cells were grown for 3 days on semipermeable membranes and treated with 1 mM dbcAMP (A), 100 nM dexamethasone (B) or a combination of 1 mM dbcAMP + 100 nM

dexamethasone (C,D) added at the apical and basolateral sides of the cells. Treatment of alveolar type II cells with dbcAMP or dexamethasone raised transepithelial current with a time course similar to  $\alpha$ ENaC mRNA expression. Potential differences across the monolayers (pd; mV) and transepithelial resistance ( $R_t$ ;  $\Omega \cdot \text{cm}^2$ ) were recorded with EVOM, at  $T_0$ , 8 h, 24 h, 32 h and 48 h of treatment and transepithelial current was determined as reported. The data are presented as percentages of current compared to time-matched controls. The effect of each type of treatment was measured in triplicate for each time point, and the whole procedure was repeated 3 times with alveolar cells isolated from different animals.  $\star p < 0.05$  (unpaired t-test): Panel A: 8 h and 32 h, 0 h and 8 h, 8 h and 48 h; Panel B: 0 h and 24 h, 24 h and 32 h, 24 h and 48 h; Panel C: 0 h and 8 h, 8 h and 32 h, 8 h and 48 h. To confirm that EVOM is sensitive enough to detect the modulation of transepithelial current in dbcAMP + dexamethasone-treated cells, measurements were also performed in the Ussing chamber (D). With both methods, treated cells show a marked increase in short circuit current  $I_{SC}$  compared to untreated cells. Although  $I_{SC}$  recording with Ussing is more sensitive in detecting current variations, the difference is not statistically significant between EVOM and Ussing measurements (t-test;  $n=5$  filters for Ussing and  $n=14$  filters for EVOM). dbcAMP: dibutyl cAMP.

#### DESCRIPTION OF THE PREFERRED EMBODIMENT

##### 1. Alveolar epithelial type II cell isolation and culture

Alveolar epithelial type II cells were isolated by enzymatic tissue digestion with elastase (Worthington Biochemical Co., Freehold, NJ, USA) from male Sprague-Dawley rats weighing 175-200 g and were purified by a differential adherence technique in rat IgG-coated bacteriologic plastic plates (26). The cells were maintained in minimum essential medium (MEM;

Gibco/BRL, Mississauga, Ontario, Canada) containing 10% fetal bovine serum (FBS, Gibco), 0.08 mg/L gentamicin, 0.2% NaHCO<sub>3</sub>, 0.01 M HEPES and 2 mM L-glutamine, and were plated at a density of  $4 \times 10^5$  cells/cm<sup>2</sup> in plastic cell culture flasks (25 cm<sup>2</sup>). They were then cultured in a humidified incubator at 37°C with 5% CO<sub>2</sub>, and the medium was replaced every 2-3 days.

## 2. Northern blotting

Total RNA from alveolar type II cells was extracted by a modification of the guanidinium-phenol technique (16;21). Cells growing in 25-cm<sup>2</sup> flasks were lysed directly on plates with 600 µl solution D and extracted as reported elsewhere (21). Ten µg of total RNA were electrophoresed on 1% agarose-formaldehyde gel and transferred to GeneScreen nylon membranes (NEN, Boston, MA, USA) by overnight blotting with 10 X SSC. Hybridization was performed, as reported previously (21), in Church buffer (0.5 M Na phosphate, pH 7.2, 7% SDS (w/v), 1 mM EDTA, pH 8) (17). Blots were exposed to Kodak Xar-film, using an intensifying screen, or to a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA) for densitometric analysis. The nylon membranes were hybridized successively with different cDNA probes ( $\alpha$ ENaC,  $\alpha_1$ Na<sup>+</sup>-K<sup>+</sup>-ATPase,  $\beta$ -actin, 18S). Between each round of hybridization, the membranes were stripped by treatment with 0.1 X SSC, 1% SDS and 2.5 mM EDTA at 95°C. The blots were allowed to cool gradually with agitation for 30 min at room temperature. The membranes were then rinsed with 5 X SSC and rehybridized.

## 3. Cloning and sequencing of the mouse $\alpha$ ENaC gene

To determine the nature of the regulatory sequence that could drive  $\alpha$ ENaC gene expression, we screened a mouse genomic library

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kindly provided by Drs. A. Reaume and R. Zimhigbi (6). This library was produced by Sau3A partial digestion of genomic DNA from a 129-Sv mouse ligated into BamHI-cut  $\lambda$  DASH II vector (Stratagene, La Jolla, CA, USA). One  $\times 10^6$  plaques were probed with 2 mouse  $\alpha$ ENaC cDNAs ( $\alpha$ mENaC nt 76 - 1676;  $\alpha$ mENaC nt 1333 - 2097) (21) according to standard procedures (59). A single 20-kb DNA was isolated. From this clone, a 3228-bp BamHI fragment, which strongly hybridized to  $\alpha$ rENaC nt 0 - 223 probe (21), was subcloned into pBluescript KS (Stratagene) and sequenced by the dideoxy technique (60) (Pharmacia Biotech, Baie d'Urfé, Québec, Canada) in the presence of DMSO, using the T3 and T7 primers of the vector. The full sequence of 3-kb BamHI genomic DNA was determined with a set of nested deletions generated by digestion with exonuclease III in the Erase-a-base system (Promega, Madison, WI, USA) following the protocol provided by the company. A computer search for putative regulatory elements of the mouse  $\alpha$ ENaC promoter was undertaken with GeneWorks software (Intelligenetics, Mountain View, CA, USA) and transcription element search software (TESS) by J. Schug and G.C. Overton of the Computational Biology and Informatics Laboratory, School of Medicine, University of Pennsylvania (URL: <http://agave.humgen.upenn.edu/tess/index.html>).

#### 4. 5'-RACE

The transcription initiation site was determined by 5'-RACE from RNA purified from the CD1 mouse lung and kidney. Twenty  $\mu$ g of total RNA were heated for 5 min at 65°C and kept on ice for 3 min. cDNA was synthesized with 62.5 U AMV reverse transcriptase (Boehringer Mannheim, Laval, Québec, Canada), using 500 pmoles of random dNTP hexamere (Pharmacia) as primer (31), and incubated for 60 min at 55°C in reverse transcription buffer (50 mM Tris-HCl, pH 8.5, 8 mM MgCl<sub>2</sub>, 30 mM KCl, 1 mM

DTT) with a mixture of dNTPs (1 mM each) and human placental RNase inhibitor (25 U) (Life Technologies, Burlington, Ontario, Canada). After 10-min incubation at 65°C, the cDNA was purified over a G-50 Sephadex spun column, dried by lyophilization and resuspended in 19 µl sterile H<sub>2</sub>O. Tailing of deoxyadenosine 5'-triphosphate (0.3 mM) was performed in 100 mM potassium cacodylate, pH 7.2, 1 mM CoCl<sub>2</sub>, and 0.1 mM DTT with 30 U of terminal transferase (Life Technologies) for 1 h at 37°C followed by 10-min incubation at 70°C. For PCR amplification, 20 pmoles (0.4 µM) of the sense primer 5'-GGA ATT CTC GAG ATC GAT GCT T<sub>(16)</sub>-3' containing an EcoRI site, 50 pmoles (1 µM) of the sense primer 5'-GGA ATT CTC GAG ATC GAT GCT and 50 pmoles (1 µM) of the antisense primer 5'-CGG GAT CCT TGC ATG GGC AGA GGA GGA C-3' corresponding to nt 191 to 211 of the αENaC gene linked to a BamHI site were used as described (21). The PCR conditions were 35 cycles of 1 min 94°C denaturation, 1 min 52°C annealing, and 2 min 72°C extension followed by a 7-min final extension at 72°C. RACE DNA was digested with BamHI and EcoRI and ligated to the corresponding sites in pBluescript KS (Stratagene).

#### 5. Transient transfection and CAT assay

For the reporter gene assay, a BamHI-MscI 2.9 kb fragment of the mouse αENaC genomic clone was linked upstream of the chloramphenicol acetyl transferase gene (CAT) in pJfCAT vector (a gift from Dr. Pierre-André Bédard, York University, North York, Ontario, Canada) at a Msc I site 161 nt upstream of the ORF but within 5'-untranslated region (5'UTR) downstream of the first transcription initiation start site. For transfection, the plasmid was purified with the QIAEX II kit (QIAGEN, Mississauga, Ontario, Canada) according to the manufacturer's protocol. A549 cells were a generous gift from Dr. André Cantin (Pneumology Division, Medecine Department,

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Université de Sherbrooke). The cells were cultured in DMEM, % FBS in the presence of penicillin (50u/ml) and streptomycin (50 µg/ml). The day before transfection,  $8 \times 10^5$  cells were seeded in 60-mm dishes and cultured for 24 h to reach 80% confluency. 6 µg of plasmid (4 µg of ENaC/CAT plasmid and 2 µg pSV-β-galactosidase) mixed with 12 µl of transfection reagent (Superfect, IAGEN) were incubated for 10 min at 21°C in 150 µl of culture medium devoided of antibiotic and serum. The DNA complex was added to cells in 1 ml culture medium (complete with FBS and antibiotic) and incubated for 3 h at 37°C. 48 h after transfection, the cells were collected, resuspended in 40 µl 250 mM Tris-HCl at pH 8 and lysed by 3 cycles of freeze-thawing (5 min dry ice-ethanol/5 min 37°C). Chloramphenicol acetyl transferase (CAT) assay and β-galactosidase assay were performed as described (59;76). After pelleting the cell debris by centrifugation (13, 000 RPM) and heating the supernatant for 10 min at 65°C, 20 µl of the cell lysate were incubated with 2 µl 200 µCi/ml [<sup>14</sup>C]chloramphenicol, 20 µl 4 mM acetyl CoA, 32.5 µl 1 M Tris-HCl, pH 7.5, and 75.5 µl H<sub>2</sub>O for 1 h at 37°C. Acetylated chloramphenicol was removed by extraction with 2 volumes of TMPD/xylene by vigorous shaking, and the top organic phase was counted by scintillation in a β-counter (1). β-Galactosidase activity of the cell lysate was quantified (59).

#### 6. Electrophysiology

For electrophysiological study, 2 techniques were employed. Since we wanted to evaluate the long-term effects of dbcAMP and dexamethasone in most experiments, potential differences across the monolayers ( $P_d$ ; mV) and transepithelial resistance ( $R_t$ ;  $\Omega/\text{cm}^2$ ) were measured with an epithelial voltohmeter (EVOM) (World Precision Instruments, Inc., Sarasota, FL, USA). At select times after the initiation of treatment (0 h, 8 h, 24 h, 32 h, 48 h). Treatment of the cells, plated at a

density of  $1.0 \times 10^6$  cells/cm<sup>2</sup> on permeable membranes (Costar Transwell, Toronto, Ontario, Canada, Catalog No. 3401, 1.0 cm<sup>2</sup>), was started after the cells reached confluency with 3 days of culture at 37°C in a humidified atmosphere and 5% CO<sub>2</sub>. Transepithelial current across these monolayers was calculated by the following formula:  $I_t = pd / R_t$ .

To validate and confirm the modulation of transepithelial current via EVOM measurements, we also determined the impact of treatment that produced a maximal effect (dbcAMP + dex 8 h), using  $I_{SC}$  assessment in the Ussing chamber. For this validation,  $I_{SC}$  was evaluated 8 h after the initiation of treatment with dbcAMP 1 mM and dexamethasone 100 nM. The filters (0.33 cm<sup>2</sup>) were placed in a special adapter and mounted in Lucite half chambers (MRA International, Naples, FL, USA). Warm (37°C) MEM supplemented with 10% FBS was circulated across both faces of the filter by gas-lift oxygenation. The transepithelial potential difference was clamped to zero by an external current passing circuit, and the resulting  $I_{SC}$  was recorded continuously on a chart recorder.  $R_{tE}$  was determined from the current needed to clamp the voltage from 0 to 1 mV for 1 sec every 10 sec (Dual epithelial voltage clamp, Warner Instrument Corporation, Hamden, CT, USA).

#### 7. Statistics

The data are presented as means  $\pm$  SE. Comparisons between groups were analyzed by unpaired t-test or analysis of variance (ANOVA) and post hoc comparison (Fisher PLSD), depending on the experimental design. A probability ( $p$ ) of  $< 0.05$  was considered to be significant.

#### Experimental protocols

*Modulation of  $\alpha$ ENaC and  $\alpha_1$ Na<sup>+</sup>-K<sup>+</sup>-ATPase mRNA expression by dbcAMP and dexamethasone*



The effects of dbcAMP and dexamethasone were tested on  $\alpha$ ENaC and  $\alpha_1$ Na<sup>+</sup>-K<sup>+</sup>-ATPase mRNA expression in alveolar type II cells. These cells were cultured for 3 days, at which time they were forming a tight epithelial monolayer and were able to perform vectorial sodium transport, allowing the formation of domes. The cells were then treated for periods of 1 h, 4 h, 8 h, 24 h or 48 h with 1 mM dbcAMP or 100 nM dexamethasone in medium supplemented with 10% FBS. A 1 mM concentration was chosen for dbcAMP because it is known to increase the activity and synthesis of Na<sup>+</sup>-K<sup>+</sup>-ATPase in alveolar type II cells (43;68). The 100-nM dexamethasone concentration was chosen because it has been shown to stimulate ENaC expression in fetal distal lung epithelial cells (12) as well as in cultured fetal lung explants (73). For each incubation period, total RNA was extracted, and the amount of  $\alpha$ ENaC and  $\alpha_1$  Na<sup>+</sup>-K<sup>+</sup>-ATPase mRNA was quantified by Northern blotting. To detect  $\alpha$ ENaC mRNA, the blots were hybridized with 764-bp mouse  $\alpha$ ENaC cDNA (His-445 to stop codon) which has a high homology with rat  $\alpha$ ENaC cDNA (21). The Northern blots were also hybridized with cDNA for  $\alpha_1$ Na<sup>+</sup>-K<sup>+</sup>-ATPase (52), a gift from Dr. J. Orłowski (Physiology Department, McGill University, Montreal, Quebec, Canada), which consists of a NarI-StuI 332-bp fragment coding from nt 89 to 421 (from the 5'UTR to Arg-61) of the rat kidney and brain  $\alpha$  isoform (64). For quantitative study,  $\alpha$ ENaC mRNA expression was normalized to  $\beta$ -actin expression to ensure that the same amount of RNA was present on each lane. The  $\beta$ -actin probe, a gift from Dr. P. Hamet (Centre hospitalier de l'Université de Montréal-Hôtel-Dieu, Montréal, Québec, Canada), consists of a Pst-I 1.5-kb cDNA fragment coding for rat brain  $\beta$ -actin (47).

The expression of  $\alpha$ ENaC and  $\alpha_1$ Na<sup>+</sup>-K<sup>+</sup>-ATPase mRNA detected in treated cells was always compared to matched control cells for each time point of the study. To ensure that the results were not being

affected by serum, the experiments were repeated with dbcAMP in serum-free media. We could not find differences in the modulation of  $\alpha$ ENaC mRNA expression under 10% FBS versus serum free conditions. For reproducibility and statistical reasons, Northern blotting was repeated several times with RNA extracted from cells isolated from different animals (n)(with dbcAMP: 1 h, n=4; 4 h, n=7; 8 h, n=18; 24 h, n=8; 48 h, n=8; with dexamethasone: 1 h, n=10; 4 h, n=13; 8 h, n=14; 24 h, n=10; 48 h, n=10).

*Effect of conjoint treatment with dbcAMP and dexamethasone on  $\alpha$ ENaC and  $\alpha_1$ Na<sup>+</sup>-K<sup>+</sup>-ATPase mRNA expression*

To determine if dbcAMP and dexamethasone could have additive or synergistic effects on the expression of  $\alpha$ ENaC and  $\alpha_1$ Na<sup>+</sup>-K<sup>+</sup>-ATPase mRNA, alveolar type II cells cultured for 3 days were treated for 8 h with 1 mM dbcAMP, 100 nM dexamethasone or a combination of both agents. mRNA was quantified by Northern blotting as described above. The experiments were repeated several times with alveolar cells isolated from different animals (dbcAMP, n=18; dexamethasone, n=12; dbcAMP + dexamethasone, n=3).

*Role of transcription and translation in the modulation of  $\alpha$ ENaC and  $\alpha_1$ Na<sup>+</sup>-K<sup>+</sup>-ATPase mRNA expression by dbcAMP and dexamethasone*

We then studied the impact of transcription on the modulation of  $\alpha$ ENaC and  $\alpha_1$ Na<sup>+</sup>-K<sup>+</sup>-ATPase mRNA expression in dbcAMP- and dexamethasone-treated cells. Alveolar cells cultured for 3 days were treated for 8 h with 1 mM dbcAMP or 100 nM dexamethasone in the presence or absence of the transcription inhibitor actinomycin D (5  $\mu$ g/ml). Because actinomycin D was prepared as a 200X (1 mg/ml) stock solution diluted in 95% ethanol, a similar amount of ethanol was added to control cells. The importance of translation in the modulation of  $\alpha$ ENaC and  $\alpha_1$ Na<sup>+</sup>-K<sup>+</sup>-ATPase mRNA expression was also tested. Alveolar cells cultured for 3 days were

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treated for 8 h with 1 mM dbcAMP or 100 nM dexamethasone in the presence or absence of 2.5 µg/ml cycloheximide.  $\alpha$ ENaC and  $\alpha_1$ Na<sup>+</sup>-K<sup>+</sup>-ATPase mRNAs were quantified by Northern blotting as described earlier. Because actinomycin D stops the transcription of all RNA, including the  $\beta$ -actin transcript, we chose to normalize the amount of RNA loaded on the gel with 18S rRNA, a very abundant RNA in our preparation, using 640-bp DNA generated by RT-PCR between nt 852 and 1492 of the rat 18S rRNA sequence (13). In the quantitative study, the treatments were repeated at least 3 times in alveolar type II cells from different rats. For the determination of  $\alpha$ ENaC mRNA half-life, alveolar type II cells were cultured on 6-well plates (9 cm<sup>2</sup>) seeded with 2.16 X 10<sup>6</sup> cells cultured for 3 days. The cells were treated with 5 µg/ml actinomycin D for 0, 2, 4, 6, 8 or 10 h in the presence or absence of 1 mM dbcAMP or 100 nM dexamethasone. RNA was purified with Trisol reagent according to the manufacturer's protocol (GIBCO/BRL, Burlington, Ontario, Canada) and quantified by Northern blotting. 18S rRNA was used to normalize the amount of RNA loaded on each well. Four different experiments were combined to generate the mRNA decay slope.

*Activity of the mouse  $\alpha$ ENaC promoter in A549 cells*

A549 cells were transfected at least 4 different times in duplicate.  $\beta$ -Galactosidase activity was used to normalize the differences in transfection efficiency arising from plate to plate. CAT activity was reported in arbitrary units consisting of the <sup>14</sup>C cpm of the sample minus the <sup>14</sup>C cpm of untransfected cells.

*Impact of dbcAMP and dexamethasone on transepithelial current*

To study the time course of transepithelial current change brought about by dbcAMP and dexamethasone, the cells were grown for 3 days on a semipermeable membrane and treated with 1 mM dbcAMP, 100 nM

dexamethasone or a combination of 1 mM dbcAMP + 100 nM dexamethasone added on their apical and basolateral sides. Potential differences across the monolayers (pd; mV) and transepithelial resistance ( $R_{te}$ ;  $\Omega \cdot \text{cm}^2$ ) were recorded with EVOM at  $T_0$ , 8 h, 24 h, 32 h and 48 h of treatment. The effect of each type of treatment was measured in triplicate for each time point, and the entire procedure was repeated 3 times with alveolar cells isolated from different rats. To determine the amount of amiloride-sensitive current, alveolar type II cells were grown as described or treated for 8 h with 1 mM dbcAMP and 100 nM dexamethasone. Pd and  $R_{te}$  were measured successively for each filter (n=15 from 5 animals) with EVOM following 5-min incubation at 37°C with 1  $\mu\text{M}$ , 10  $\mu\text{M}$  and 100  $\mu\text{M}$  amiloride. For  $I_{sc}$  measurement in the Ussing chamber, the cells were grown as described and treated for 8 h with 100 nM dexamethasone and 1 mM dbcAMP (n=5 filters from 2 animals).

## RESULTS

### *Modulation of $\alpha\text{ENaC}$ and $\alpha_1\text{Na}^+-\text{K}^+-\text{ATPase}$ mRNA expression by dbcAMP and dexamethasone*

To investigate the effect of dbcAMP and dexamethasone on  $\alpha\text{ENaC}$  and  $\alpha_1\text{Na}^+-\text{K}^+-\text{ATPase}$  mRNA expression, alveolar type II cells were treated after 3 days of culture when they reached confluency, were forming a tight epithelial monolayer, and were capable of vectorial sodium transport resulting in the formation of domes. DbcAMP modulated  $\alpha\text{ENaC}$  and  $\alpha_1\text{Na}^+-\text{K}^+-\text{ATPase}$  mRNA expression similarly in these cells (Fig. 1A, 1B). There was a 2-fold transient increase in the level of  $\alpha\text{ENaC}$  and  $\alpha_1\text{Na}^+-\text{K}^+-\text{ATPase}$  mRNA, reaching a maximum after 8 h of treatment (Fig. 1A, 1B).  $\beta$  and  $\gamma\text{ENaC}$  mRNAs were also upregulated by dbcAMP but at a lesser extent than for  $\alpha\text{ENaC}$  mRNA. (data not shown). A good correlation was obtained between  $\alpha\text{ENaC}$  and  $\alpha_1\text{Na}^+-\text{K}^+-\text{ATPase}$  mRNA expression in cells treated

with dbcAMP ( $R=0.889$ ).

Dexamethasone, on the other hand, differentially modulated  $\alpha$ ENaC and  $\alpha_1$ Na<sup>+</sup>-K<sup>+</sup>-ATPase mRNA expression (Fig. 1C, 1D). There was a gradual increase in  $\alpha$ ENaC mRNA, with maximum expression (4.4-fold rise) occurring after 24 h of treatment (Fig. 1C).  $\beta$  and  $\gamma$ ENaC mRNAs were also upregulated by dexamethasone (data not shown). Dexamethasone had no significant effect on  $\alpha_1$ Na<sup>+</sup>-K<sup>+</sup>-ATPase mRNA expression (Fig. 1D). No correlation was found between  $\alpha$ ENaC and  $\alpha_1$ Na<sup>+</sup>-K<sup>+</sup>-ATPase mRNA expression in dexamethasone-treated cells ( $R=0.390$ ).

*Effect of conjoint treatment with dbcAMP and dexamethasone on  $\alpha$ ENaC and  $\alpha_1$  Na<sup>+</sup>-K<sup>+</sup>-ATPase mRNA expression*

To determine if conjoint treatment with dbcAMP and dexamethasone could have an additive effect on  $\alpha$ ENaC and  $\alpha_1$ Na<sup>+</sup>-K<sup>+</sup>-ATPase mRNA expression, alveolar type II cells were treated for 8 h with 1 mM dbcAMP, 100 nM dexamethasone or a combination of the 2 agents. Densitometric quantitation of Northern blots showed that cells treated with dbcAMP and dexamethasone presented a statistically significant increase in  $\alpha$ ENaC and  $\alpha_1$ Na<sup>+</sup>-K<sup>+</sup>-ATPase mRNA expression (Fig. 2) compared to cells treated with either agent alone. This increase was additive for  $\alpha_1$ Na<sup>+</sup>-K<sup>+</sup>-ATPase mRNA expression (3.3 X) or slightly more than additive for  $\alpha$ ENaC mRNA expression (6.6 X).

*Role of transcription and translation in the modulation of  $\alpha$ ENaC and  $\alpha_1$ Na<sup>+</sup>-K<sup>+</sup>-ATPase mRNA expression by dbcAMP and dexamethasone*

We then investigated the role of transcription and translation in the modulation of  $\alpha$ ENaC and  $\alpha_1$ Na<sup>+</sup>-K<sup>+</sup>-ATPase mRNA expression by dbcAMP and dexamethasone. The transcription inhibitor actinomycin D (5  $\mu$ g/ml) abolished the increase of  $\alpha$ ENaC mRNA detected after 8 h of treatment with dbcAMP or dexamethasone (Fig. 3A, Table I). It

also diminished the increase of  $\alpha_1\text{Na}^+\text{-K}^+\text{-ATPase}$  mRNA seen in dbcAMP-treated cells (Fig. 3A, Table I). The translation inhibitor cycloheximide (2.5  $\mu\text{g/ml}$ ) decreased the basal level of  $\alpha\text{ENaC}$  mRNA expression by 75% (Fig. 3B, Table I). However, it did not abolish the increase in  $\alpha\text{ENaC}$  mRNA brought about by dexamethasone (Fig. 3B, Table I). Although the level of  $\alpha\text{ENaC}$  mRNA expression in dbcAMP + cycloheximide-treated cells was 56% of the untreated control value, dbcAMP in these cells still doubled the expression of the gene compared to cycloheximide treatment alone (Table I). The basal expression level of  $\alpha_1\text{Na}^+\text{-K}^+\text{-ATPase}$  transcripts was not decreased by cycloheximide. However, cycloheximide inhibited the increase in  $\alpha_1\text{Na}^+\text{-K}^+\text{-ATPase}$  mRNA evoked by dbcAMP (Fig. 3B, Table I).

To test if the elevated  $\alpha\text{ENaC}$  steady state mRNA level brought about by dbcAMP and dexamethasone was not caused by mRNA stabilization, the half-life ( $T_{1/2}$ ) of  $\alpha\text{ENaC}$  RNA was determined. The half-life of  $\alpha\text{ENaC}$  mRNA in untreated cells was very long (15.1 h) and followed logarithmic decay (Fig. 4A). Neither dbcAMP ( $T_{1/2}$  of 13.8 h; Fig. 4B) nor dexamethasone ( $T_{1/2}$  of 10.4 h; Fig. 4C) increased it.

#### *Cloning of the 5'-flanking region of mouse $\alpha\text{ENaC}$ gene*

Although the promoter of  $\alpha_1\text{Na}^+\text{-K}^+\text{-ATPase}$  gene has been cloned and characterized (65;67), the  $\alpha\text{ENaC}$  promoter has been cloned only recently (37;63;70). To determine the nature of the regulatory sequence that could drive  $\alpha\text{ENaC}$  gene expression, a mouse genomic DNA library was probed with 2 mouse  $\alpha\text{ENaC}$  cDNAs:  $\alpha\text{mENaC}$  nt 76 - 1676;  $\alpha\text{mENaC}$  nt 1333 - 2097 (21). A 3.2-kb BamHI fragment, consisting of part of exon I comprising the start of translation (ATG), the 5'UTR and 2.7 kb of the 5'-flanking sequence, was cloned and sequenced (Fig. 5). Several potential transcription initiation sites were found by 5'-RACE for lung and kidney RNA (Fig. 5). No TATA box or CCAAT box was seen. Numerous GRE half sites

and 1 progesterone receptor (GRE/PR) were found (Figs. 5 and 6). Only 1 GRE at position -718 to -732 bp of the first potential transcription site had the proper orientation and spacing to be a functional GRE (Fig. 5). Two consensus sequences for ATF/CREB transcription factors at positions -1756 and +572 bp of the longer transcription site could modulate cAMP transcription of the gene (Fig. 5). A CREB/c-jun binding site was also detected at position -1695 bp. Other consensus sequences for the binding of AP-1, AP-2, Sp1, Ets, GATA-1 and PEA3 transcription factor were also seen with a degenerate NF- $\kappa$ B (NF- $\kappa$ B deg) (Fig. 5).

**Activity of the mouse  $\alpha$ ENaC promoter in A549 cells**

To test the activity of the mouse  $\alpha$ ENaC promoter, a BamHI-MscI 2.9 kb-fragment linked to a CAT reporter gene was transfected in A549 lung epithelial cells. The fragment encompasses 2.45 kb of the sequence upstream to the first transcription initiation start site (Fig. 6A). Although CAT expression driven by the promoter was modest, it was statistically different from cells transfected with the p $\beta$ gal vector alone ( $p < 0.05$ ) (Fig. 6B). Treatment with dbcAMP for 8 h caused non-significant changes in the expression of the CAT gene compared to untreated cells (Fig. 6B). Dexamethasone treatment for 24 h increased CAT activity in these cells by a factor of 5.9 (Fig. 6B).

***Impact of dbcAMP and dexamethasone on transepithelial current***

To determine if changes in  $\alpha$ ENaC and  $\alpha_1$ Na<sup>+</sup>-K<sup>+</sup>-ATPase mRNA expression have an impact on ion transport, we measured Pd and R<sub>t</sub> with EVOM and calculated the transepithelial current of alveolar type II cells grown on permeable filters treated with dbcAMP, dexamethasone, or a combination of the 2 agents (Fig. 7). There was a significant increase of transepithelial current 8 h after the beginning of treatment with dbcAMP (Fig. 7A) or dbcAMP + dexamethasone (Fig. 7C). This heightened transepithelial

current decreased gradually toward control values over 48 h. Dexamethasone also induced an increase of transepithelial current that was smaller than with combined treatment and reached its maximum at 24 h (Fig. 7B). The transepithelial currents determined after 8h of treatment for stimulated and unstimulated cells were mainly amiloride-sensitive with a 61 to 74% inhibition with 10  $\mu$ M amiloride (table II). To confirm the modulation of transepithelial current detected with EVOM, the  $I_{SC}$  of cells treated for 8 h with dbcAMP and dexamethasone was recorded with the Ussing system. Treated cells showed a marked increase in  $I_{SC}$  compared to untreated cells (Fig. 7D).

#### Discussion

In this study, we investigated the hypothesis that modulation of  $\alpha$ ENaC and  $\alpha_1$ Na<sup>+</sup>-K<sup>+</sup>-ATPase gene expression could be a mechanism that regulates vectorial sodium transport in alveolar type II cells. We demonstrated that cAMP and dexamethasone both increased  $\alpha$ ENaC mRNA expression in adult alveolar type II cells and that gene transcription was involved in this elevation. We found parallel modulation of  $\alpha_1$ Na<sup>+</sup>-K<sup>+</sup>-ATPase and  $\alpha$ ENaC mRNA with dbcAMP but not with dexamethasone, which had no impact on  $\alpha_1$ Na<sup>+</sup>-K<sup>+</sup>-ATPase mRNA expression.

Treatment of alveolar type II cells with 1 mM dbcAMP modified both  $\alpha$ ENaC and  $\alpha_1$ Na<sup>+</sup>-K<sup>+</sup>-ATPase mRNA expression. The expression of both mRNAs doubled transiently after 8 h of treatment before returning to control values after 24 h (Fig. 1A,1B). Forskolin (20  $\mu$ M), an activator of adenylate cyclase which increases cytosolic cAMP, gave similar results (data not shown), demonstrating that cAMP and not butyrate modulated the level of  $\alpha$ ENaC and  $\alpha_1$ Na<sup>+</sup>-K<sup>+</sup>-ATPase mRNA. This regulation involved the transcription of new mRNAs since the elevated level of transcript promoted by dbcAMP was sensitive to actinomycin D. Because dbcAMP could increase the steady state level of  $\alpha$ ENaC mRNA in alveolar type II cells



by enhancing the stability of the transcripts, we determined the half-life of  $\alpha$ ENaC mRNA in these cells. We found that  $\alpha$ ENaC mRNA had a long half-life (15, 1 h), longer than the half-life reported in the parotid gland (8 h)(78) but shorter than the half-life in fetal distal lung epithelial cells (22 h)(53). DbcAMP did not augment the stability of the transcript, showing that transcription of the gene is involved in increasing the steady state level of  $\alpha$ ENaC mRNA (Fig. 3A).

The correlation of  $\alpha$ ENaC and  $\alpha_1$ Na<sup>+</sup>-K<sup>+</sup>-ATPase mRNA expression ( $R=0.889$ ) suggests that cAMP co-regulates the amount of  $\alpha$ ENaC and  $\alpha_1$ Na<sup>+</sup>-K<sup>+</sup>-ATPase mRNA in alveolar type II cells. However, the mechanisms involved in upregulation of the 2 genes are probably different. The heightened of  $\alpha$ ENaC mRNA expression generated by dbcAMP was not inhibited by cycloheximide, suggesting that cAMP can directly stimulate the expression of the gene whereas the  $\alpha_1$ Na<sup>+</sup>-K<sup>+</sup>-ATPase mRNA increase was sensitive to cycloheximide, indicating that the effect of cAMP was mediated by the secondary synthesis of some transcription factor (Fig. 3, Table I). One unexpected finding was that cycloheximide downregulated basal  $\alpha$ ENaC mRNA expression in alveolar type II cells (Fig. 3B). Such an observation has also been reported for  $\alpha$ ,  $\beta$  and  $\gamma$ ENaC mRNA in cultured human fetal lung explants (73). The inhibition of expression elicited by cycloheximide is not unique to ENaC since other genes are affected similarly (18;27;42). This inhibition suggests that a short-lived labile factor could be important for the basal transcription or stability of  $\alpha$ ENaC mRNA.

Dexamethasone markedly affected  $\alpha$ ENaC mRNA expression in alveolar type II cells, augmenting it by 3-fold after 8 h of treatment and by 4.4-fold after 24 h, with values remaining elevated after 48 h (Fig. 1C). Transcription of new mRNA was involved in the increment of  $\alpha$ ENaC mRNA with dexamethasone since it was abolished by actinomycin D

(Fig. 3B). Dexamethasone did not increase the half-life of  $\alpha$ ENaC mRNA, suggesting transcription of new mRNA and not enhanced stability of the transcripts. Although dexamethasone has been shown to modulate  $\alpha$ ENaC mRNA in fetal lungs and cells (49;69;73), in adult rat lungs following adrenalectomy (56) and in a bronchial epithelial cell line (36), the results reported here are the first to confirm that it augments  $\alpha$ ENaC mRNA expression in adult alveolar type II cells. As for dbcAMP, the data with cycloheximide indicate that dexamethasone directly stimulates transcription of the gene and do not require the translation of another gene product.

In contrast to  $\alpha$ ENaC, dexamethasone did not alter  $\alpha_1$ Na<sup>+</sup>-K<sup>+</sup>-ATPase mRNA expression in alveolar type II cells. This is in agreement with several reports showing that dexamethasone does not increase  $\alpha_1$ Na<sup>+</sup>-K<sup>+</sup>-ATPase mRNA expression in alveolar type II cells (2) or in the fetal lung during development (34;69). However, it has been demonstrated recently that dexamethasone elevates the expression of  $\alpha_1$ Na<sup>+</sup>-K<sup>+</sup>-ATPase mRNA in a rat pre-type II cell line (FD18) (11). Furthermore, Barquin et al. (2) have shown that dexamethasone can upregulate transiently after 12 h of treatment the  $\beta_1$ Na<sup>+</sup>-K<sup>+</sup>-ATPase expression. This leads also to transient increase in Na<sup>+</sup>-K<sup>+</sup>-ATPase activity (2). These differences could be related in part to the fact that experiments were conducted in fetal cell line (11) and that different time points were studied (2). It is clear however that dexamethasone does not have a sustained effect on the expression or activity of Na<sup>+</sup>-K<sup>+</sup>-ATPase.

The results presented above indicate that dexamethasone and dbcAMP modulate the expression of the  $\alpha$ ENaC gene through distinct pathways. We, therefore, tested if combined treatment with dbcAMP and dexamethasone would have an impact on  $\alpha$ ENaC mRNA expression. Treatment of alveolar type II cells with 100 nM dexamethasone and 1 mM dbcAMP for 8 h had an additive effect on  $\alpha$ ENaC mRNA, raising the

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expression of these transcripts by 6.6-fold. This is different from that found in 20-24-week fetal lung explants where 8-bromo-cAMP had no impact on  $\alpha$ ENaC mRNA expression and did not further increase the heightened expression evoked by dexamethasone (73), suggesting again that the regulatory mechanism may differ between adult and fetal cells.

The profile of  $\alpha$ ENaC and  $\alpha_1$ Na<sup>+</sup>-K<sup>+</sup>-ATPase mRNA elicited by dbcAMP in alveolar type II cells shows transient activation followed by fading expression. This is reminiscent of a gene regulated by a CRE (44;62) where activation of transcription through protein kinase A is downregulated by protein phosphatase (28). The slow but strong increase of  $\alpha$ ENaC mRNA by dexamethasone suggests that GRE could modulate the expression of the gene (72). To evaluate if we could identify putative regulatory element in the promoter of ENaC, we cloned and sequenced 2.7 kb of the 5'-flanking region of mouse  $\alpha$ ENaC gene. We chose to focus on ENaC and not on  $\alpha_1$ Na<sup>+</sup>-K<sup>+</sup>-ATPase gene because the promoter was already characterized (25;65;67) and because gene expression was not modulated by dexamethasone and was not directly stimulated by dbcAMP. Recently, several investigators reported cloning of the promoter for  $\alpha$ ENaC gene in humans (63;70) and rats (53;78) as well as of the human  $\gamma$ ENaC promoter (71).

The mouse  $\alpha$ ENaC gene presents some similarities as well as differences with human  $\alpha$ ENaC gene (70). The sequence of 5'-RACE products is identical to that of the genomic clone, showing that in the mouse, as it has been demonstrated in the rat (53), the 5'UTR and start of the open reading frame (ATG) are part of exon I. This is in contrast to human  $\alpha$ ENaC gene (70) where a 665-190 nt intron has been found in conjunction with alternative splicing between some of the initiation start sites and exon 2. As for human  $\gamma$  and  $\alpha$ ENaC genes (70;71) and rat  $\alpha$ ENaC gene, several

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potential transcription initiation sites with 2 major ones at -606 and -72 bp of ATG have been identified in the mouse by 5'-RACE. These multiple transcription start sites could arise from the lack of TATA or CCAAT boxes in the 5'-flanking sequence of ENaC genes. The 5'-flanking sequence of mouse  $\alpha$ ENaC gene, as for other genes devoided of a TATA box, is very rich in GC content. It lacks, however, GC boxes and GC box homologous motifs identified for human  $\gamma$  and  $\alpha$ ENaC genes (70;71). Sp1-binding elements used in some TATA-less promoter for transcription initiation (51) are nevertheless found at 10-13 bp of some transcription initiation sites (-606, -287).

The activity of the promoter was tested in A549 cells, a cell line considered by many investigators to be representative of alveolar epithelial cells. The  $\alpha$ ENaC genomic clone acts as an active promoter driving the expression of CAT reporter gene in A549 cells. The activity of the promoter in unstimulated cells is very weak, and reflects the low basal expression of  $\alpha$ ENaC mRNA in these cells (data not shown). Upon stimulation with dexamethsone, however, there is a 6-fold increase in the activity of the promoter compared to unstimulated cells. Thus, dexamethasone modulates  $\alpha$ ENaC mRNA expression by augmenting gene transcription. Similar results have been obtained recently for the rat and human  $\alpha$ ENaC promoter (37;53;63). The -718-to -732-bp GRE that we find in the mouse  $\alpha$ ENaC promoter (AGAACA<sub>gaa</sub>TGTCCT) is identical in sequence and position, relative to ATG, to the functional GRE detected in the rat promoter (37;53). Further work must be done to determine if this site is a functional GRE in the mouse promoter.

We could not find any stimulation of CAT expression with dbcAMP. It is possible that the 2.9-kb fragment tested does not contain an active CRE. It is also possible that A549 cells differentially modulate the expression of  $\alpha$ ENaC gene compared to alveolar type II cells. By Northern

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blotting, we could not detect an increase in the steady state level of  $\alpha$ ENaC mRNA in these cells after treatment with dbcAMP. Finally, because  $\square$ -agonists and dbcAMP have been observed to increase intracellular calcium concentration  $[Ca^{++}]_i$  in alveolar epithelial cell (46;61), we can not exclude the possibility that the rise in  $[Ca^{++}]_i$  could be involved in the modulation of transcription as demonstrated for other genes (14;29;35;57).

The modulation of  $\alpha$ ENaC and  $\alpha$ 1Na<sup>+</sup>-K<sup>+</sup>-ATPase mRNA expression observed upon treatment of alveolar type II cells with dbcAMP or dexamethasone might not necessarily result in a concomitant rise in the activity of the channel or the sodium pump. Furthermore, cAMP and aldosterone have been shown to increase amiloride-sensitive sodium current in alveolar type II cells within minutes (15;19;20;74) without enhancing the synthesis of ENaC or Na<sup>+</sup>-K<sup>+</sup>-ATPase. For this reason, we tested the physiological effect of dbcAMP and dexamethasone on alveolar type II cells by recording transepithelial currents occurring after single or combined treatment. To study the impact of the synthesis of new channels on transepithelial current, we investigated the longterm effect of dbcAMP and dexamethasone. Treatment of alveolar type II cells with dbcAMP or dexamethasone increased transepithelial current with a time course similar to  $\alpha$ ENaC mRNA expression (Fig. 7) rather than Na<sup>+</sup>-K<sup>+</sup>-ATPase expression. With dbcAMP, maximum current was recorded after 8 h of stimulation whereas with dexamethasone, the maximum was reached after 24 h. Several studies have shown a parallel increase between the expression of  $\alpha$ ENaC mRNA and the transepithelial current detected in cells treated with dexamethasone (12;32) or aldosterone (40). The results presented here are the first to reveal modulation of transepithelial current concomitant with  $\alpha$ ENaC mRNA expression in alveolar type II cells treated with dbcAMP and/or dexamethasone. The preponderance of amiloride-sensitive current detected

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in stimulated and unstimulated alveolar type II cell (table II), as well as the increased steady state level of  $\beta$  and  $\gamma$ ENaC mRNA upon dbcAMP or dexamethasone treatment (data not shown) suggest that the synthesis of new ENaC channels could be an important limiting step for sodium reabsorption in lung alveolar epithelial cells as it has been proposed in other epithelial cells (58), for example kidney cells.

These results also indicate, however, that other factors besides an increase in  $\alpha$ ENaC mRNA expression are important to stimulate transepithelial current. Dexamethasone, a more potent inducer of  $\alpha$ ENaC mRNA than dbcAMP, fails to stimulate transepithelial current to the same level as dbcAMP. Its influence is also negligible on the modulation of transepithelial current induced by combined treatment with dexamethasone and dbcAMP (Fig. 7C, table II). Several explanations can be offered for these differences. First, although dexamethasone induces a higher steady state of  $\alpha$ ENaC mRNA expression than dbcAMP, it is possible that synthesis of the protein might not correlate with the level of ENaC mRNA detected. A second explanation might be that an increased expression of  $\alpha$ ENaC is not sufficient *per se* to stimulate  $\text{Na}^+$  transport. DbcAMP and not dexamethasone could stimulate via protein kinase A other factors that increase channel activity. Finally, it is also possible that besides ENaC, an increase in the expression or activity of  $\text{Na}^+-\text{K}^+-\text{ATPase}$  is also required to augment sodium transport in alveolar epithelial cells. DbcAMP produces a concomitant elevation of ENaC and  $\alpha_1\text{Na}^+-\text{K}^+-\text{ATPase}$  mRNA expression, whereas dexamethasone has little impact on  $\alpha_1\text{Na}^+-\text{K}^+-\text{ATPase}$  expression in our study (Fig. 1) and had only transient effect on the expression of the  $\beta_1$  subunit of  $\text{Na}^+-\text{K}^+-\text{ATPase}$  and sodium pump activity (2). This could possibly explain the absence of a strong rise in transepithelial current in dexamethasone-treated cells. This hypothesis is supported by the fact that in the intact lung, during edema resolution there

is not only an elevation of ENaC (5) but also  $\alpha_1\text{Na}^+\text{-K}^+\text{-ATPase}$  synthesis (5). The importance of  $\text{Na}^+\text{-K}^+\text{-ATPase}$  expression in sodium transport has been demonstrated recently in gene therapy where enhanced  $\text{Na}^+\text{-K}^+\text{-ATPase}$  expression in the lung augments lung liquid clearance (23;66). It is also possible that an increase in  $\text{Na}^+\text{-K}^+\text{-ATPase}$  activity rather than its heightened expression is needed to raise amiloride-sensitive transepithelial current. In this regard, we have shown that chronic stimulation with dbcAMP does lead to enhanced activation of  $\text{Na}^+\text{-K}^+\text{-ATPase}$  (43). In lung epithelial cells, unlike kidney epithelial cells, the modulation of  $\text{Na}^+\text{-K}^+\text{-ATPase}$  synthesis and activity in parallel to changes in ENaC could play an important role in transepithelial sodium transport. In summary, we found that dbcAMP affects the expression of  $\alpha\text{ENaC}$  and  $\alpha_1\text{Na}^+\text{-K}^+\text{-ATPase}$  mRNAs in alveolar type II cells whereas dexamethasone only modulates the expression of  $\alpha\text{ENaC}$  mRNA. Treatment with actinomycin D and mRNA decay study have shown that the increase in  $\alpha\text{ENaC}$  mRNA comes from an increase in gene transcription, and not from stabilization of  $\alpha\text{ENaC}$  mRNA. Cloning of the mouse  $\alpha\text{ENaC}$  promoter and testing of its activity have demonstrated that regulatory elements present in the 2.7-kb 5'-flanking sequence allow the regulation of transcription by dexamethasone. There is a parallel increase in the expression of  $\alpha\text{ENaC}$  and  $\alpha_1\text{Na}^+\text{-K}^+\text{-ATPase}$  mRNA produced by dbcAMP treatment and the transepithelial current recorded in alveolar type II cells. Clearly, transcription of  $\alpha\text{ENaC}$  and  $\alpha_1\text{Na}^+\text{-K}^+\text{-ATPase}$  mRNA is part of the mechanisms that regulate sodium transport in alveolar cells.

In view of the foregoing, it is readily apparent that a cell capable of expressing a reporter gene, which expression is governed by the ENaC promoter, would be a valuable tool for screening pharmacological compounds capable of affecting the activity of the promoter. Sodium absorption by the epithelial sodium channel is involved in important

physiological functions. Therefore, in first instance, the ENaC gene promoter sequence is an object of the present invention. In second instance, a construct which comprises the ENaC gene promoter and a reporter gene is another object of this invention. In third instance, a vector comprising the construct and capable of effecting the task of transfecting the construct into the host cell and integrating the construct into cellular genome, is another object of this invention. In fourth instance, a host cell comprising, stably integrated in its genome, the above construct (promoter and reporter gene), is further another object of this invention.

Many reporter genes are known and provide a convenient way by which the activity of a promoter can be evaluated. These reporter genes are "cassettes" that are introduced by homologous or non-homologous recombination into host cells expressing ENaC by techniques well-known to the skilled artisan. The host cells will originate from different epithelial tissues, for example, alveolar II cells, cells from diverse kidney anatomic sections, and any other epithelial cells of interest. Epithelial cells of different anatomic structures do not express the same cell machinery; so different extracellular receptors to drugs, hormones or growth factors are harbored at their surface. Further, different transcription factors are involved in different cellular pathways, depending on cascades of events triggered by the binding of a ligand (drugs, hormones or growth factors) to a receptor, and on the transduction signals operating and leading to a physiological effect upon ligand-receptor complexing. There is therefore a need for evaluating the effect of drugs, hormones or growth factors on different epithelia, to evaluate 1) potential pharmaceutically active compounds, 2) their tissue specificity and selectivity, 3) their positive or negative effect on the promoter activity, and 4) the transcription factors involved. Consequently, it is a fifth object of this invention to provide a method for screening pharmaceutical compounds of

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interest which comprises the steps of: testing the compounds with a recombinant host cell comprising ENaC promoter and a reporter gene, and measuring the expression of the reporter gene as an indication of an effect of the compounds.

The compounds that have a negative effect on promoter activity in kidney cells, for example, may be retained as potential candidates for treating hypertension, for example. On the contrary, compounds having a positive effect may be useful for treating pseudo-hypoaldosteronism (22,39,81). In the respiratory tract, compounds that have a positive effect on promoter activity in alveolar lung cells would be retained as candidates for treating problems of liquid clearance at birth and edema clearance in adults (4,10,45,51,55). On the opposite, compounds having a negative effect may be used for treating symptoms associated with cystic fibrosis (11 to 13), for example. Other compounds for treating epithelial diseases or disorders of diverse origin, for example, diarrhea or hyperhydrosis, are considered within the goals of the present tools and screening methods.

Example of promoter, reporter gene, recombinant vectors and host cell for the purpose of screening therapeutic compounds for respiratory diseases:

The screening of new factors that could modulate the expression of the ENaC gene in lung epithelial cells is a long and tedious process. The concentration of ENaC mRNA has to be determined by Northern Blot, RNase protection assay or quantitative RT-PCR. We propose here to generate a cellular model by performing a stable transfection of human A549 cells, a cell line with phenotypic similarities to alveolar type II cells with the ENaC promoter linked to the CAT reporter gene.

The A549 cells will be transfected with a set of ENaC promoter-CAT construct that has been found to be effective in transient

transfection of A549 cells and kidney epithelial cells to drive the expression of the CAT gene. It will be co-transfected with 1/5 the amount of pcDNA3 vector (Invitrogen) coding for the Neo<sup>r</sup> gene (aminoglycoside phosphotransferase) that allows the selection of the cells with G-418 (Geneticin, Life technologies) antibiotic. After four days to allow the expression and integration of the selective marker, the cells will be divided with a 1/10 dilution and treated with G-418 to allow the selection of the table transfectants. The optimal concentration of G-418 will have to be optimized for the A549 cell line. We will start with a 400 µg/ml concentration for selection and 200 µg/ml concentration to maintain the cell thereafter, as suggested by the manufacturer. The resistant cells colonies after 7 to 10 days of selection will be picked and the cells cloned. The presence of the ENaC genomic clone will be tested by PCR and Southern blots to verify that the promoter and CAT coding sequences are integrated before testing the CAT activity of the cells.

A549 cells are cultured in DMEM, 10% FBS in presence of penicillin (50u/ml) and streptomycin (50µg/ml). They are seeded at a density of 4000 cells/cm<sup>2</sup> in 100mm cell culture flasks and kept in a 5% CO<sub>2</sub>-air incubator at 37 C. The medium is replaced every two days. The day before transfection, 9 X 10<sup>5</sup> cells are seeded in 60-mm dish and grown for 24 hours until they reach a 80% confluency. Prior to transfection the cells are washed once in PBS before the incubation with the DNA/Superfect complex. 5µg of plasmid (4 µg of ENaC/CAT plasmid and 1 µg pcDNA3) mixed to 10 µl of transfection reagent (Superfect, Qiagen) are incubated for 10 min at 21 C in 150 µl of culture medium devoided of antibiotic and serum. The DNA complex is added to cells in 1 ml culture medium (complete with FBS and antibiotic) and is then incubated for 3 h at 37 C. After 48 hours of culture, the cells will be divided and cultured with a 1:10 dilution in presence of G-418. The appropriate amount of G-418 needed to achieve selection will have to be

determined. After 7 and 10 days the resistant cells will be picked and cloned. Different segments of the ENaC genomic DNA have already been inserted in pUMSV40CAT (73) and pJFCAT (16) vector and are ready for transfection.

The cloning and sequencing of the ENaC promoter is an important scientific step to understand the factors involved in the modulation of ENaC expression. It gives essential informations leading the research to find new ways to modulate the expression of the channel and the generation of new therapies. The generation of cell lines bearing different fragments of the ENaC promoter is essential for the commercialization of our work and is needed to take advantage of the patent. There is no doubt that such a cell line could interest pharmaceutical companies and research teams involved in ENaC modulation and provide an essential research tool for the screening and testing of compounds susceptible to modulate ENaC expression. The different plasmids bearing the ENaC/CAT construct and the cell lines bearing these constructs are new products directly protected by the patent that could be offered to the marked of bio/pharmaceutical companies.

Although the present invention has been described hereinabove by way of preferred embodiments thereof, it can be modified, without departing from the spirit and nature of the subject invention as defined in the appended claims.

**Table I. Expression of  $\alpha$ EnaC and  $\alpha 1$  Na<sup>+</sup>-K<sup>+</sup>-ATPase mRNA upon treatment with actinomycin D and cycloheximide**

Treatment	$\alpha$ EnaC (n)	$\alpha 1$ Na <sup>+</sup> -K <sup>+</sup> ATPase (n)
Act D	94.8 $\pm$ 12.2 (3)	83.1 $\pm$ 12.0 (3)
Act D + dbcAMP	89.0 $\pm$ 11.3 (3)	73.8 $\pm$ 16.5 (3)
Act D	100.3 $\pm$ 12.7 (5)	90.0 $\pm$ 11.2 (5)
Act D + Dex	99.6 $\pm$ 13.4 (5)	79.6 $\pm$ 8.0 (5)
Cyclo	25.1 $\pm$ 3.5 (3)	117.1 $\pm$ 11.1 (3)
Cyclo + dbcAMP	55.7 $\pm$ 7.9 (3)	115.5 $\pm$ 4.3 (3)
Cyclo	27.0 $\pm$ 2.1 (3)	138.9 $\pm$ 26.5 (3)
Cyclo + Dex	328 $\pm$ 19.5 (3)	113.8 $\pm$ 13.3 (3)

Act D: actinomycin D; dbcAMP: dibutyryl cAMP; Cyclo: cycloheximide; Dex: dexamethasone; n: number of samples. The data are presented as percentages of control after correction with 18S rRNA.

Table II. Bioelectric properties of alveolar type II cell monolayers with EVOM.

	Unstimulated	dex 8h	dbcAMP 8 h	dbcAMP + dex 8 h
PD, mV	4.43 ± 1.00	8.91 ± 0.89	13.43 ± 0.75	18.04 ± 1.14
RTE, $\Omega \cdot \text{cm}^2$	762 ± 91	1278 ± 102	1447 ± 53	1664 ± 89
I <sub>TE</sub> , $\mu\text{A}$	4.98 ± 0.70	6.83 ± 0.33	9.29 ± 0.38	10.78 ± 0.18
+ amiloride 1 $\mu\text{M}$	2.56 ± 0.17 (48.7%)	3.49 ± 0.38 (48.9%)	3.93 ± 0.28 (57.7%)	5.15 ± 0.32 (52.3%)
+ amiloride 10 $\mu\text{M}$	1.93 ± 0.13 (61.3%)	1.81 ± 0.10 (73.6%)	2.66 ± 0.11 (71.4%)	3.69 ± 0.53 (65.8%)
+ amiloride 100 $\mu\text{M}$	1.57 ± 0.07 (68.5%)	1.33 ± 0.04 (80.6%)	2.14 ± 0.11 (77.0%)	2.68 ± 0.47 (75.1%)

Alveolar type II cells were grown for three days and stimulated or not for 8 h, PD: potential difference, RTE : transepithelial resistance, I<sub>TE</sub> : Transepithelial current, (%): % of I<sub>TE</sub> inhibited by amiloride, dbcAMP : 1 mM dibutyryl cAMP, dex : 100 nM dexamethasone. n = 15 filters from 5 different animals.

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**What is claimed is:**

1. An epithelial sodium channel gene promoter having the nucleic acid sequence shown in Figure 5, a variant thereof or a fragment thereof comprising at least one of regulatory elements responding to at least one DNA-binding proteins shown in Figure 6.
2. A DNA construct comprising the promoter of claim 1 and a heterologous gene operably linked thereto.
3. The construct of claim 2, wherein said heterologous gene is a reporter gene.
4. An expression vector comprising the construct of claim 3.
5. A recombinant host cell comprising the construct of claim 3.
6. A recombinant host cell as defined in claim 5 which is an epithelial cell.
7. A recombinant host cell as defined in claim 6, wherein the host cell originates from a tissue involved in sodium reabsorption.
8. A recombinant host as defined in claim 7 wherein the host cell endogenously expresses said sodium channel.
9. A method for screening compounds to select a potentially active pharmaceutical capable of modulating the expression of an epithelial sodium channel gene, which comprises:
  - testing said compounds with a recombinant host cell as defined in any one of claims 5 to 8;
  - detecting a difference between the levels of expression of said reporter gene in the presence and in the absence of said compounds; and
  - selecting a compound which shows such a difference as

said potential pharmaceutical of interest.

10. The method of claim 9, wherein the pharmaceutical is for treating sodium and water retention.

11. The method of claim 9 wherein said host cell is a  
5 alveolar lung cell and said pharmaceutical is for treating lung edema.

12. The method of claim 9, wherein said host cell is a tubular kidney cell and said pharmaceutical is for treating hypertension.



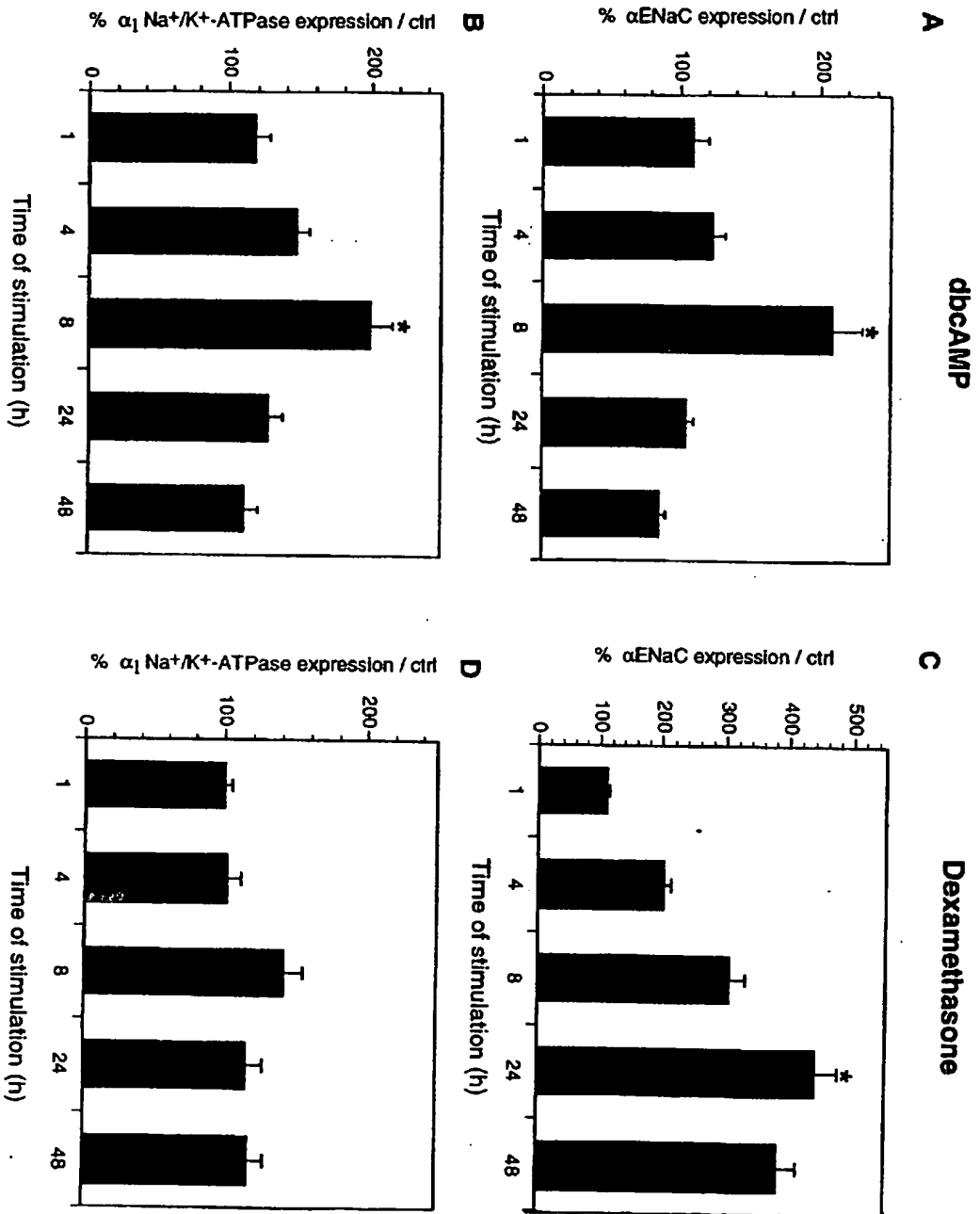


FIGURE 1

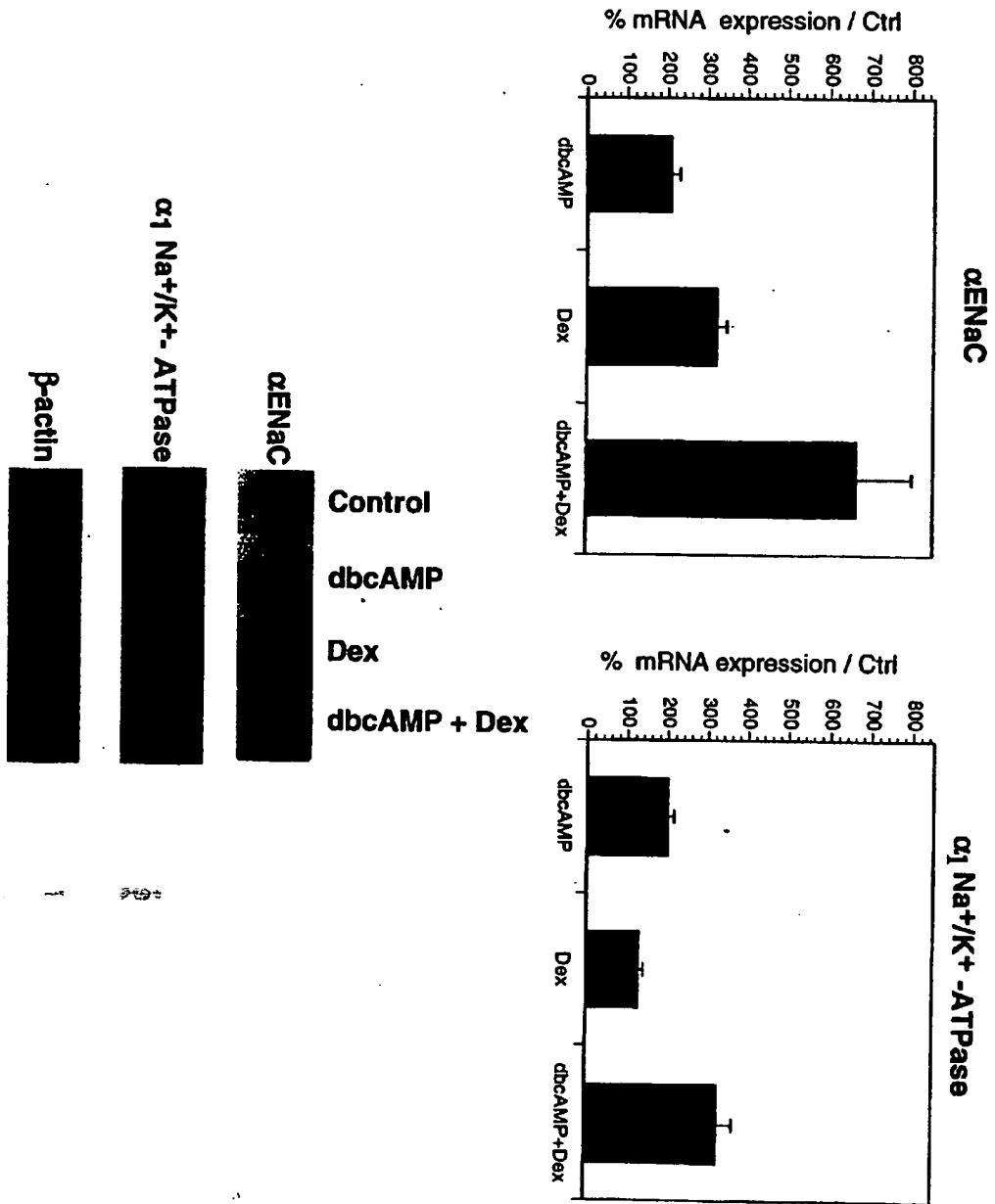
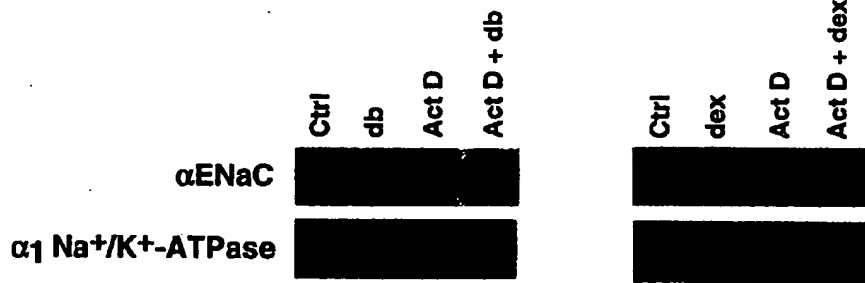


FIGURE 2

**A**



**B**

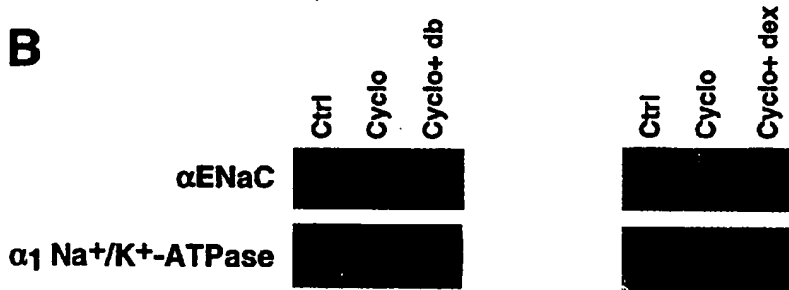


FIGURE 3

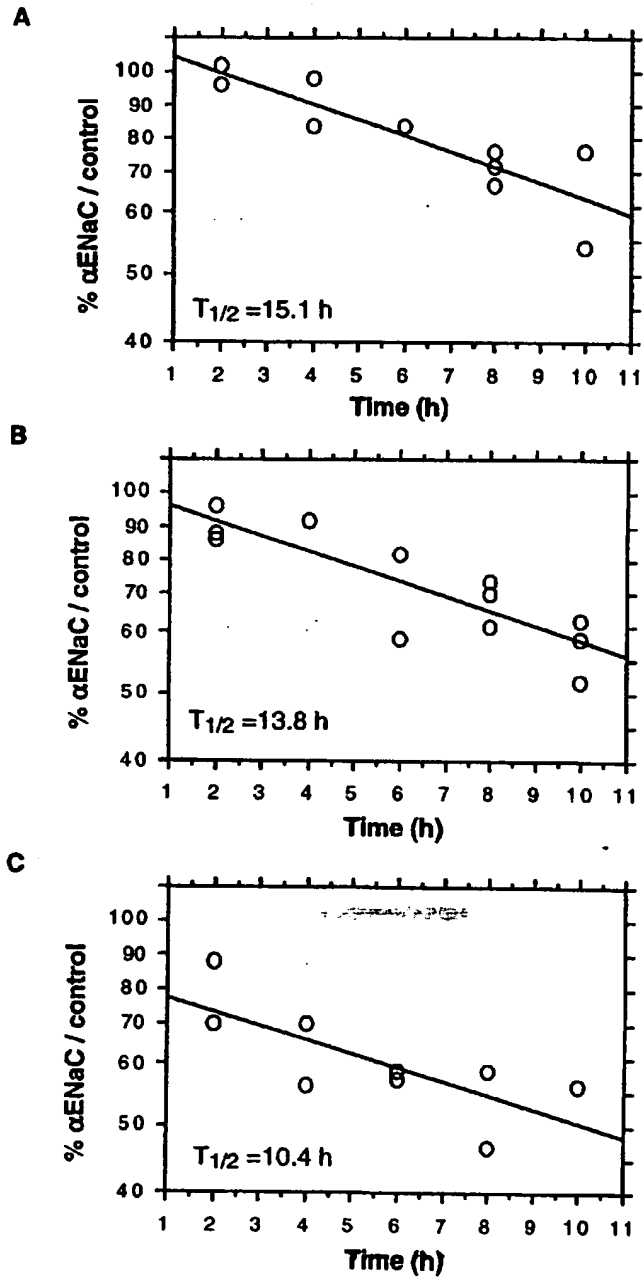
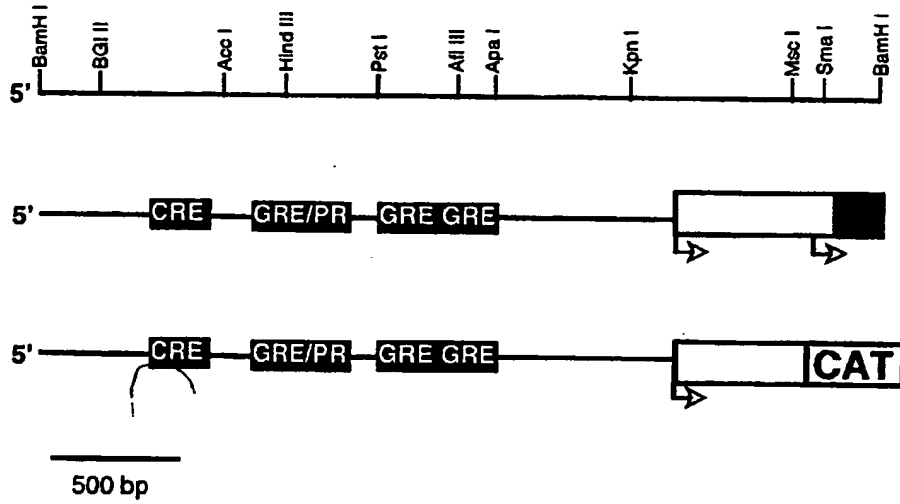


FIGURE 4

GGATCCCCTG GTTTGGTGT GTTGTGTTT GTGTGTGTGT GTGTGTATGT GTGTGTGTGT GTGTGCATGT ATTTGAATGT -2371  
CTGTCTGTCT GTCTGTCTGT CTGTCTGTAT TTGAATATCT ATGTGTATGC ATGTGTATTT GTGAGTGTGA TTCAAATGGG -2291  
ACTCTGACCA AAGTACCAAT ATCTATTCTT ACATCATGCT TGCCTGGCC CATTCAGAT CTCTAGTGCC GTGGTCACCA -2211  
CGCCAAGCCA AGCCAAGCCA CTCTCTCATA CTCTGTCAAG ACATGCCAGA GTCACCACAC TCTTTGGGCC AGGCATTCTC -2131  
ACGGAGTCTT TGAGATGAAA ACTATTGTG ATGCTGCCTT AAGCCAGTAC TTGGGTTCA GGGCCAGCTA GCAAACAGAA -2051  
GAGGCAGGGA GGAGCCAGCT TCTCTTCG GAACCTCACA GACTAAACAG TCAGGGGAAG CGGGCACCTT GCCTGTGGA -1971  
ACACTGCTTT AAGGACTTGC TTGATTCAAG GGAGCATAGG GGTGAGCTGG AATGGAG AGGTAGAAAC AGCTCAGAGG -1891  
CACACAGCCA AGGAGACATT ACCTGGAGAG GGTGAATCCA TAGGCTTTGT GTGGGAGGGA CAAAACCCCT CTGTCTGGCC -1811  
TGGTCCCCCA GAGTTATGA CTGGTCTGTC TCAGGAGGGA AATGACGAGA GGCAACGTC GAGGAAAAGA TGTTTTGTTT -1731  
GCATTCTGTC TACAACAGCT GCTGGTCCGC TTTGTATGT CAGAAAAGT GCTTATTGGT TTGGAGGGTC GGTTCCTGTG -1651  
TGTGAGAAGT GGAGAAGGAG CCTGCCTCCC TCTCTTACA GAAAGGTTTC GTGGATCTGT GTCAGGGCGT CTGTCAAACA -1571  
GCCTAGCTAA CAGGCGGCAC CCAGGTTGAC CATTCAAGA ATGAGACTAG CTCAGAGGAC CCTCTTATTT GTCTGGTTTA -1491  
TTCTGCAAGC TTCAGCCGCA ATGCCAGTCT TCAGGGTGTG GCTTGAATG AGTCCCCTCA GGGTGTCACT TGAATGGGT -1411  
CCCTCCTGTT CTTCCGCCA CTGTCCCCCA GCCTTGGTT CCTATCCCTG CCTCCAGACC CCTCATCTCC CTGTATGTCA -1331  
ACTACCTGTT GCTTTCCATA AACCTCTGTG GCTGCTTGG TCTGTGTCTG TAGGCTGGA GCACAGGAA CACCCCTGTC -1251  
TGTGTTTCCT GCTGTGGGA GAGAGGGTAG GAAGAGCCAG TGAGCACACT GGAGCACTGG GTGCCTGCC TCTCCAGGCC -1171  
TCAAGTGGCA CTATCACTAG CAAGCTCTGC AGGACCTGG CTATGTGGAT GGCTCTCCA TGACTCTCA CTCTTCTTT -1091  
TGGGGGTGTG AGCCTTGAG GTGTACACC TGTGTGTCC CTGCTGCAT CTGTCTCTC TAGCTAGGCT AGCTAGCTTG -1011  
AGGGAGAGTT TGGAGAAAA CACAGCCTGG ATGAAAGAGC TTCCACTTCT TTCTAGGACA GCGTCCCAC CCTCTTGGC -931  
CTAGATACCA GTATACACAC TCTTGTCTCTCTCCAGG GTCTGCCATC CCTGCCTGTC CAGGGCTCTT GGTCTGTGCC -851  
CACAGAGCTC TGCCTGGCCC CCATGTACAT GTTACCTCTG GTGCCACAT TCTTGCAACC CTGTGACC AACAGGGGAC -771  
ATTACACATT CCTGGCTAT CAGCCAATAG TGTCAAAAG AACGAAATGT CCTAGGACCT GGCCAGCCC CCTACTTCAC -691  
CTGGGCCCTT CCTCCAGG CTTGGACAGG GCCAGGTAGA TGGGGTGA GAAGGGGAG GGGGAGAAGA -611  
TGGTGGGCTC CAGCAGGTGC TCCAGTTTT GGGGACCCA TTCTCTTTT CCTCCAGGAC CTTGGGTGTG AGGCTGACCT -531  
GGGATGACCT TGAGGGACCT CAATCAGAAG GGACCCTGGT TTCTGAAAT TCACCTAGAT TTCCCTTCCC CTAGCCATCT -451  
TGCTGACAG GAGAGTCCCT TTGGAGCTGG CTCCTCTC TTCTCTCTC TTCACTCTCC CTCAGCTCTT TGCACAGTCC -371  
TCCCTAGTCT GTCTCTTTT TCTGCTTCC CTGTCTCTT GGTTCCTCC CCTACTCTCG GCGATCTCC TTCCGCTTCT -291  
GCCCTCCTGG CTTTGGCGTG TAGAATCCTT CCTCTCTCT CCGGTCTCT GAGCCTCAC TGCTCTCTGC CCCTAGTCTC -211  
TTTTCAGTC AGTTCGCCAT CAATCCCTGG CCGAACTCT TCAGCAGGTA CCTGGTTCAC CTGTCCCCCA AGAGCCCTCC -131  
TCCCTGGAGA GCTCCAGAC CAGACTCTC CTCGATCCT CCTCTGCCC TGCTCACCTT TAATTGAGT GCTAATGAGG -51  
CTTCTGTC TCCATCCTT GCCCGTGGC GACGGGCGT CTCTGGAGCC AGGCACTGCA CCTGTCAGGT GAGAGGGTGG 30  
AGAGGCTCGG CTGCCAGATT TAACTGGAAA GGAACAGTC ACAGCCAGC CACACTGGA GCGGGAGCA GGAGGCAGCT 110  
CCGGCTCTT GCAACCCAGG GTCCCGAGG CAGAGAAGGA GGTAGCAGG AGCTGGAGG CAGGGCTAGA GAGCCTAGAG 190  
AAGAGGCC AGGAGCAGAT AGGAAGSCA GGAAGGAAG TGAGGCAGGA TCAGAGACC TGGCACAGG AGGGAGACC 270  
AAAGAGAAG GAGAGTASC TGGCCAAAG GGGCGTAAA GCCGGAGAT CAACAGTCC GGGAGGAAA AGGGGCAAGA 350  
GGGAGAGGC GCTAAGCCAG ACAGGGTGC TGTGTGGAG ACCCAGGAG GCGTAGCGG GCAAACGAAG GTGGCCTCG 430  
CTGTGACACT TCGCTCTGTG GCCACTCCAG TGAAGCTCCG TGCTGCCTGG TTGGCCCAA CTCCAGAAGG TCAGCTGGCT 510  
CCTGGAGAGG TGGAGGAGG TGGAGGACT CAGGAAGAGG GAACTCAGCG TGGATGCGG GCTCCTCAG GACGGCCCA 590  
TTCTGCCTCC ATACTAATGA TGCTGGACCA CACCAGAGCC CCTGAGCTCA ACCTGACCT AGACCTTGAC GTCTCCAAT 670  
CACCAGAGG ATCCATGAAG GGCACAATT TCAAGGAGCA AGACTTTGT CCTCTCTGC CCAATGCAAGG ACTGGGGAG 750  
GGGACAAGCG TGAAGAACAG GCGTGGG 778

FIGURE 5

**A**



**B**

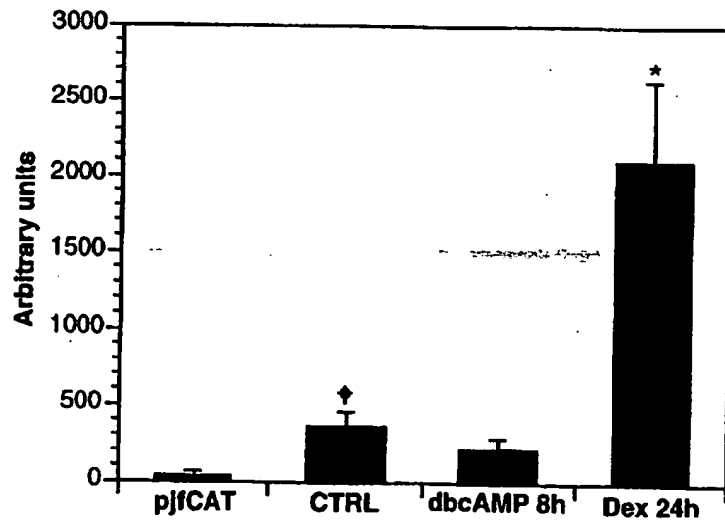


FIGURE 6

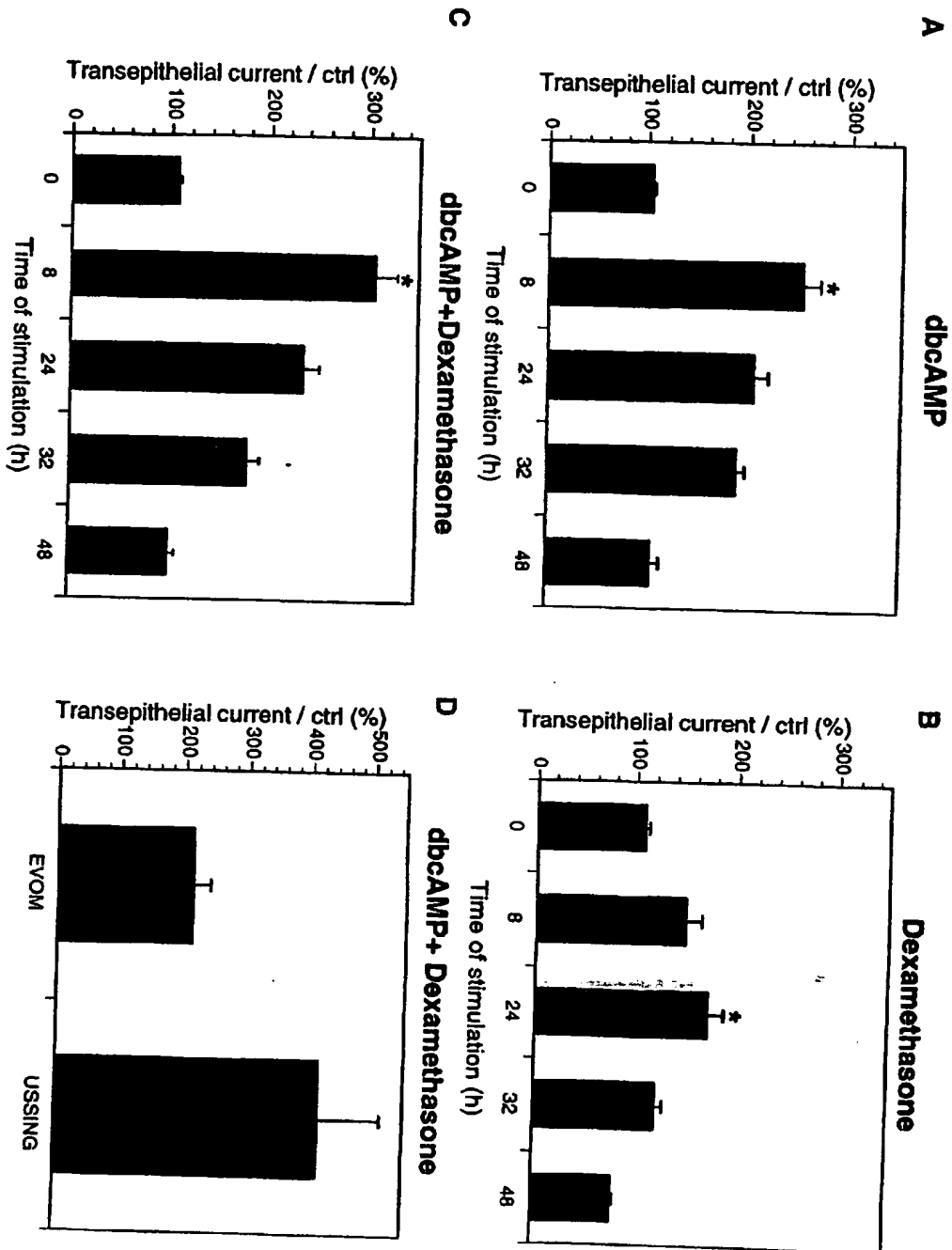


FIGURE 7

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